REMARKS

Claims 1-18 and 21-22 are pending. Claims 1, 7, 11, 14 and 15 are amended herein. Claim 1 is amended to shorten the preamble of the claim. Claims 7 and 11 are amended to more clearly outline the scope of the claims. Claims 14 and 15 are amended in view of the antecedent basis provided in the independent claim.

I. REJECTION OF CLAIMS UNDER 35 U.S.C. § 112, SECOND PARAGRAPH

Claims 7, 11, and 14-15 are rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. In particular, the Examiner alleges that there is no antecedent basis for various terms in these claims. In response and solely for the purposes of expediting prosecution of the instant application, Applicants have herein amended claims 7 and 11 by deleting reference to a precipitating agent. Further claims 14 and 15 are amended by deleting reference to the coagulant and naming thrombin. In view of the foregoing, withdrawal of the rejection under 35 U.S.C. §112, second paragraph is respectfully requested.

II. REJECTION OF CLAIMS UNDER 35 U.S.C. §103(a)

A. GRAY & COCHRUM

Claims 1-3, and 7-15 remain rejected under 35 U.S.C. §103(a) as being unpatentable over Gray *et al.* (U.S. Patent No. 4,680,177; hereinafter "Gray") in view of Cochrum *et al. (U.S.* Patent No. 5,773,033; hereinafter "Cochrum"). In particular, the Examiner points to col. 4, lines 39-49 of Gray, alleging Gray teaches a method for the production of blood products wherein anticoagulated whole blood or blood plasma is processed by cryoprecipitation to yield a precipitate that is separated from the supernatant. While acknowledging that Gray discloses blood plasma as preferred, the Examiner alleges that whole blood is indicated as an option in Gray.

A (i) GRAY

Gray discloses blood products and derivatives, such as procoagulants, plasma proteins, and various leucocytes, that are obtained from shed blood or its tissue precursor (bone marrow) that has been anticoagulated by the use of a neutral salt that does not bind calcium ions. Gray teaches that the preferred anticoagulants are salts containing the divalent ions calcium, magnesium, barium or strontium. Gray discloses that the salts of calcium and magnesium are especially preferred because of their relatively lower toxicity, the salt of choice being a magnesium salt, for instance magnesium chloride, because magnesium salts exhibit the required anticoagulating action at levels compatible with retention of normal ionic strength and osmolarity.

Gray also discloses that blood anticoagulated and preserved by neutral salts is qualitatively different from conventionally anticoagulated and preserved blood because the high levels of neutral salt in the blood preclude its direct clinical use as whole blood in humans, owing to the toxic effects of such high levels of neutral salt ions, for example, calcium or magnesium ions (see column 3, lines 24-31.) Gray further teaches a process that "...involves a cryoprecipitation step, that is by freezing plasma to low temperature and then thawing at 0° to 4° C..." to obtain a "...precipitate highly enriched in Factor VIII (known as cryoprecipitate or Factor VIII concentrate)..." (col. 4, lines 31-34)

Gray, therefore, teaches 1) the use of toxic levels of neutral salts as an anticoagulant for whole blood rather than conventional calcium-binding anticoagulants (for example, CPD, ACD or EDTA; and 2) consistent with what is known in the art, cryoprecipitation is performed on plasma, not whole blood.

A (ii) COCHRUM

The invention as disclosed in Cochrum is directed to autologous fibrinogen and chitosan containing hemostatic adhesive agents having strong hemostatic properties when applied to a bleeding wound or vessel. Cochrum discloses that fibrinogen is isolated and purified using ammonium sulphate precipitation in slow incremental portions. Cochrum claims a method for attaining hemostasis by administering to a site of injury a hemostatic agent consisting essentially

of a mixture of fibrinogen isolated from plasma and purified of other plasma proteins and a biocompatible chitosan polymer, wherein a ratio of the chitosan polymer to the isolated and purified fibrinogen is from about 0.1:10% to about 90:99.9%, w/w, and wherein said fibrinogen is separated from other plasma proteins by precipitation with ammonium sulfate solution having concentration of about 65-73 g per 100 ml, said precipitation comprising the addition of about 10 ml of said ammonium sulfate solution to about 40 ml of plasma continuously in increments from about 0.5 to about 0.62 ml per minute for about 15-20 minutes.

A (iii) GRAY IN VIEW OF COCHRUM

There is nothing in Gray that would lead one of skill in the art to practice the present invention as claimed. In particular, Gray fails to teach, *inter alia,* a method for the production of thrombin from anticoagulated whole blood, comprising: obtaining a volume of anticoagulated whole blood from a subject; mixing said anticoagulated whole blood with ethanol at room temperature; incubating the mixture at room temperature for a time sufficient to produce a cellular and specific plasma component precipitate and a supernatant; separating the precipitate from the supernatant; and recovering the supernatant wherein said supernatant contains a thrombin preparation comprising 80-90% of prothrombin-thrombin proteins, no detectable fibrinogen and 20-30% of baseline levels of anti-thrombin III (ATIII).

Rather, Gray relates primarily to the advantage of using neutral salts that do not bind calcium as anticoagulants in the collection of whole blood as opposed to conventional use of calcium-binding anticoagulants. Gray is cited in the Office Action for its teaching that anticoagulated whole blood can be fractionated by cryoprecipitation. Yet, Applicants respectfully highlight that Gray does not teach cryoprecipitation of whole blood without a plasma isolation step, nor is it exemplified. Rather, Gray teaches a process for isolation of certain rare blood components by a cryoprecipitation step, that is, by freezing plasma to low temperature and then thawing at 0°C to 4°C. (col. 4, lines 30-33). Despite the Examiner's assertion that the text of lines 9-21 in col. 4 teach that separation of plasma from cellular components is preferred and that whole blood is clearly indicated as an option, one of skill in the art would recognize that cryoprecipitation is only ever performed on plasma and there is no apparent

reason that one can glean from Gray for substituting a chemical method of precipitation (as in present invention) for cryoprecipitation (as in Gray), particularly where the starting material is whole blood.

Gray, therefore, fails to teach three elements of Applicants claimed method: 1) anticoagulation of whole blood with conventional Ca-binding anticoagulants; 2) precipitation of whole blood; and 3) precipitation with ethanol.

Cochrum does not remedy the deficiencies of Gray. Like Gray, Cochrum relates to a method for obtaining purified isolated fibrinogen from plasma. Cochrum teaches that autologous fibrinogen is prepared from the patients own blood which is *first separated into plasma, platelets and blood cells* (col. 10, lines 25-32) *(i.e.* not using whole blood as in the present invention). The plasma is further processed by precipitation of fibrinogen under very stringent conditions using ammonium sulfate where only fibrinogen is precipitated and there is no precipitation of other plasma proteins. (col. 7, lines 53-56; col. 10, lines 6-10; col. 10, lines, 41-60.). Like Gray, Cochrum does not teach precipitation 1) of whole blood 2) with ethanol. Thus, Cochrum does not teach or suggest, either alone or in combination with Gray, the method of producing thrombin as presently claimed.

MPEP 2143 recites seven rationales that may support a conclusion of obviousness: (1) combining prior art elements according to known methods to yield predictable results; (2) simple substitution of one known element for another to obtain predictable results; (3) use of known technique to improve similar devices (methods or products) in the same way; (4) applying a known technique to a known device (method or product) ready for improvement to yield predictable results; (5) "obvious to try" - choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success; (6) known work in one field of endeavor may prompt variations of it for use in either the same field or a different one based on design incentives or other market forces if the variations are predictable to one of ordinary skill in the art; (7) some teaching, suggestion, or motivation in the prior art that would have led one of ordinary skill to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention. With this in consideration, Applicants respectfully argue that the

combination of Gray and Cochrum does not render the instant claims as obvious, as the combination fails to support any of these rationales.

A PHOSITA would not have recognized that the results of the combination were predictable, and indeed there would have been no reasonable expectation of success to arrive at the present invention by combining the teachings of Gray and Cochrum. Applicants respectfully point out that a prior art reference must be considered in its entirety, *i.e.,* as a whole. *W.L. Gore & Associates, Inc. v. Garlock, Inc.,* 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied,* 469 U.S. 851 (1984). Applicants acknowledge that Gray teaches cryoprecipitation *(i.e.* freezing to low temperature and then thawing at 0°C to 4°C (col. 4, lines 30-33)) as a step in the isolation of certain blood components that are present in low concentrations and are difficult to isolate, however, the mere fact that Gray discloses cryoprecipitation to isolate certain blood components does not teach or suggest in any way the method of producing thrombin as taught and claimed herein, which explicitly includes use of whole blood rather than plasma *(see* claim 1 and discussion *supra).* Moreover, there is no teaching, suggestion, or motivation in the cited references or in the prior art that would have led one of ordinary skill to modify Gray's cryoprecipitation method or to combine such teaching with Cochrum's disclosure of a method for obtaining fibrinogen from plasma to arrive at the instantly claimed invention, which includes the step of mixing *whole blood* with ethanol. Therefore, for at least this reason, Cochrum cannot therefore compensate for the deficiencies in the teachings of Gray.

Whole blood (present invention) and plasma (prior art) represent distinctly different starting materials. As indicated in the Declaration of Dr. Sherwin Kevy (of record in the present application), in the event that the person of skill would even be motivated to use whole blood in the first place, he/she would have known that precipitation of an anticoagulated whole blood preparation would result in a preparation containing significant levels of cell debris and cellular proteins not present in a similarly processed plasma preparation and therefore, would likely require different handling from plasma. As detailed above, there is nothing in Cochrum that remedies the deficiencies of Gray because, *inter alia,* as is customary in the art for purifiying fibrinogen, Cochrum's method discloses isolating fibrinogen from *plasma (i.e.* not whole blood).

Applicants respectfully bring to the Examiner's attention that there are many methods for fractionating blood. The kind and conditions of precipitation affect the product obtained.[2] Cryoprecipitation is not interchangeable with precipitation with a salt, such as ammonium sulfate, which is not interchangeable with precipitation with an organic material, such as ethanol. One cannot extrapolate a method for one protein to another unrelated protein. The examiner's position that optimization of conditions would be a matter of routine is unfounded. Because there is an infinite number of combinations and permutations of parameters *(e.g.* volume of starting material used, volume of precipitating agent or method used, temperature at which the method is performed, amount of contaminants/inhibitors remaining in product, *etc.),* one of skill in the art would *not* have had a reasonable expectation of success in obtaining the desired end product simply by substituting one precipitation method for another. This is particularly true if other variables are introduced, for example, using whole blood as the starting material rather than plasma. Thus, there is no apparent reason why one of skill in the art would combine the teachings of Cochrum and Gray and achieve Applicants invention, as currently claimed.

Applicants respectfully highlight that one cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention. In re Fritch, 972 F.2d 1260 (Fed. Cir. 1992). Using the inventor's success as evidence that one of ordinary skill in the art would have reasonably expected success represents an impermissible use of hindsight. Life Technologies, Inc. v. Clontech Laboratories, Inc. 224 F.3d 1320 (Fed. Cir. 2000). It is impermissible to engage in a hindsight reconstruction of the claimed invention by using the applicant's structure as a template and selecting elements from references to fill in the gaps. In re Gorman, 933 F.2d 892 (Fed. Cir. 1991).

The Federal Circuit recently held that "[a] broader independent claim cannot be nonobvious where a dependent claim stemming from that independent claim is invalid for obviousness." (No. 2009-1076, slip op. 21-22) That is, if a dependent claim is obvious, then the parent independent claim is obvious. The logical contrapositive of this holding is that if a parent independent claim is nonobvious, then all dependent claims are nonobvious. Callaway Golf v.

[2] See Section II C on Inherency and Obviousness on page 18 of this submission.

Acushnet, 2009-1076 (Fed. Cir. 2009).

For the reasons stated above, claims 1-3, and 7-15 are not rendered obvious by Gray in view of Cochrum. Accordingly, withdrawal of the rejection under U.S.C. §103(a) is respectfully requested.

B. COELHO & ROCK

Claims 1-4, 7-18 and 21-22 remain rejected under 35 U.S.C. §103(a) as being unpatentable over Coelho *et al.* (U.S. Patent No. 6,472,162; hereinafter "Coelho") in view of Rock (U.S. Patent No. 4,359,463; hereinafter "Rock"). In particular, the Examiner points to claim 17 and alleges, *inter alia,* that Coelho teaches a method for extracting and then dispensing thrombin, said method consisting of taking whole blood from a person, sequestering prothrombin from the whole blood by addition of ethanol and converting prothrombin to thrombin.

B (i) COELHO

Coelho's invention is directed to a sterile method for preparing a stable thrombin component from a single donor's plasma in which the thrombin component and the clotting and adhesive proteins component are harvested simultaneously from the same donor plasma in less than one hour. It is clearly an object of Coelho to provide a new and novel apparatus and method to derive fast acting, stable autologous thrombin from the donor's plasma. (col. 6, lines 44-47) The combined components provide an improved biological hemostatic agent and tissue sealant by virtue of its freedom from the risk of contaminating viruses or bacteria from allogenic human or bovine blood sources. The thrombin provides polymerization of the clotting and adhesive proteins in less than five seconds, and is sufficiently stable to provide that fast clotting over a six hour period. Further, the clotting times can be predictably lengthened by diluting the thrombin with saline.

B (ii) ROCK

Rock's disclosure is directed to a method whereby initial Factor VIII activity (normally

present in blood collected into an anticoagulant which functions by chelating calcium) is maintained for twenty-four or more hours without the necessity of the usual immediate freezing in either the whole blood or blood plasma. The method comprises mixing freshly collected blood or blood plasma prepared from that blood or freshly obtained blood plasma obtained by plasmapheresis, which blood or blood plasma has been collected into a calcium chelating anticoagulant, with a calcium-heparin solution in sufficient quantities to restore calcium to substantially normal physiological levels.

B (iii) COELHO IN VIEW OF ROCK

Coelho teaches "preparing a fraction enriched in prothrombin by use of Ethanol to substantially enhance the concentration of prothrombin and at the same time remove or denature naturally occurring ingredients within *plasma,* such as Fibrinogen and Antithrombin III which can bind to, block, interfere with or inhibit prothrombin or its subsequent activation to long-term functional thrombin" (col. 6, lines 27-33). This statement clearly identifies the fraction in which prothrombin is enriched by use of ethanol as a plasma fraction. Consistent with the interpretation of the language in claim 17 "sequestering prothrombin from the whole blood by addition of ethanol," throughout the specification, Coelho refers to the sequestration of prothrombin and subsequent derivation of autologous thrombin *from plasma,* not from whole blood (abstract; col. 6, lines 27-30; col. 6, lines 44-47; col. 7, lines 10-16; col. 7, lines 38-40; col. 9, lines 13-17). Notably, nowhere does Coelho, in claim 17 or anywhere else, disclose that ethanol is added to or mixed "with whole blood." Rather, Coelho in claim 17, states the "addition of ethanol" but neglects to specify to what ethanol is added. However, the Coelho specification clearly discloses that ethanol is added to plasma, not whole blood.

Likewise, the description of Coelho's device for obtaining the thrombin repeatedly refers to plasma and not whole blood (col. 9, lines 7-10, lines 36-38, lines 47-50 *etc.).* Coelho fails to provide any evidence from which one of skill in the art would conclude that precipitation of whole blood was either desirable or advantageous for the preparation of autologous thrombin.

The Examiner relies on claims 17, 55, 97, 99, 103, 107, 112, and 116 for the position that

thrombin is isolated from whole blood. Each of these claims recite in one form or another a method for extracting autologous thrombin from a patient, the steps consisting (or consisting essentially) of obtaining whole blood from a person; sequestering plasma from whole blood; converting the prothrombin to thrombin; loading the thrombin into a syringe and using the syringe to dispense the thrombin to stem blood flow (see for e.g. claim 17 of Coelho reproduced *infra)*.[3] Nowhere, however, do any of these claims recite *mixing whole blood* with ethanol. Rather, the claims fail to teach or suggest the mixing of ethanol "with whole blood." Both the Coelho claims and specification specifically teach preparation of "a fraction enriched in prothrombin by use of ethanol to substantially enhance the concentration or prothrombin ...within plasma", not whole blood.

Furthermore, even if claim 17 could be read as teaching addition of ethanol to whole blood, which it does not, it is Applicants' position, that the specification of Coelho contains no suggestion or guidance from which one of skill in the art, using his/her own knowledge of whole blood fractionation, would conclude that whole blood and plasma are interchangeable in the Coelho method. For example, claim 17 of Coelho does not recite a step for removing the inevitable precipitate of cell debris and cellular protein components that result from mixing ethanol with whole blood, a phenomenon that does not occur with plasma since the blood's cellular component has already been removed (*see* footnote 3). Moreover, Coelho's specification is silent as to why one would omit such a step. Coelho's disclosure of a method of extracting thrombin from whole blood without a precipitate removal step is, at best, inconsistent with the teachings of the specification discussed above. According to Coelho, the purpose of the step of adding ethanol is to enrich prothrombin in the plasma and at the same time remove fibrinogen from the prothrombin, and to eliminate antithrombin III, which inhibits the formation of thrombin from prothrombin and which may inactivate the thrombin.

Coelho's specification, while it contains ample guidance with respect to the method of

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[3] Claim 17 recites: a method for extracting and then dispensing thrombin, the steps consisting of: taking whole blood from a person, sequestering prothrombin from the whole blood by addition of ethanol, wherein ethanol is present at a concentration between about 8% and about 20% volume per unit volume, converting the prothrombin to thrombin, loading the thrombin into a syringe, and using the syringe to dispense the thrombin to stem blood flow. Applicants suggest that the specification as filed was not intended to encompass claims to a method of extracting thrombin from whole blood. The numerous references in the specification to *plasma* suggest that only the plasma embodiment was contemplated at the time the application was filed.

using plasma as a starting material, is silent as to any additional considerations taking into account the significant differences between plasma and whole blood. The skilled artisan would recognize that whole blood and plasma represent completetly different starting materials. Whole blood is a complex mixture of cells and extra-cellular constituents that remain relatively unaltered upon collection of the blood with an anticoagulant. 35-45% of the volume of whole blood is composed of red blood cells; 35% of the red blood cell is hemoglobin (Kevy Declaration ¶13, of record). Mechanical or chemical disruption of the red blood cells in whole blood, for example by precipitation, generates considerable undesirable cell debris and of hemoglobin release *(see* paper enclosed herein entitled "Interference of Blood Cell Lysis on Routine Coagulation testing" *Arch Pathol Lab Med.* 2006;130:181-184).

Furthermore, in response to a previous Office Action, Applicants argued that at the time of the invention by Applicants, isolation of plasma from whole blood prior to further processing was standard in the art for preparing fibrin sealant materials from blood. In support of this position, Applicants submitted three documents to establish the state of the art at the time of the invention.

Firstly, a Declaration Under 37 CFR § 1.132 was submitted to establish that at the time the present application was filed, one of skill in the art would have recognized that precipitation of an anticoagulated whole blood preparation would result in a preparation containing significant levels of cell debris and cellular proteins not present in a similarly processed plasma preparation (Declaration on record of Sherwin V. Kevy, M.D. June 13, 2007, paragraph 12) and that prior to 2006, no report of a method of producing thrombin using whole blood without the plasma isolation step had been made. The standard of practice in the art for production of thrombin from whole blood included a plasma isolation step for the removal of cells/cell debris prior to precipitation of protein components, leaving soluble thrombin in the supernatant.

Additionally, Applicants submitted in support of the state of the art an article by ThermoGenesis (owner of the Coelho patent) scientists, Kumar and Chapman, (JECT 39:18-23, 2007, a duplicate copy of which is enclosed for the Examiner's convenience) that reports generating autologous human thrombin from whole blood as the starting material (abstract). The Kumar reference, published well after the present application was filed, represents the first

disclosure of that which Applicants had already invented. The abstract of the Kumar article states:

> "Thrombin-based clotting agents currently used for topical hemostasis with absorbable sponges, fibrin sealants, and platelet gels *are primarily derived from bovine or pooled human plasma sources...The* goal of our research was to develop a rapid, reliable, and simple to perform process to generate autologous human thrombin in the intraoperative setting, from *patient whole blood as the starting source material."* [emphasis added]
>
> "In this study, we have developed a reliable technique to generate autologous human thrombin in the intra-operative setting from *whole blood instead of plasma as the starting source material* within a 30-minute period." [emphasis added]

In Applicants' view, the Kumar and Chapman reference represents the first disclosure other than Applicants', of a method for extracting thrombin from whole blood.

Applicants respectfully urge the examiner to reconsider the Declaration of Dr. Kevy, the after-arising Kumar reference, and the brochure submitted previously that outlines the manner of using the device to perform the method of Coelho. The combination of the arguments made herein, along with the other evidence already of record, when taken together establish that, at the time of the disclosure by Coelho of a method for extracting thrombin from blood, the skilled artisan would not have thought it feasible, let alone advantageous, to precipitate plasma proteins without first removing blood cells *(i.e.* the skilled artisan would have been taught away from using whole blood).

There is nothing in the prior art to substantiate the assertion that one of skill in the art, at the time of Coelho, would have read that document as teaching a method for the extraction of autologous thrombin by the direct precipitation of whole blood. Applicants respectfully point out that while Coelho's specification contains sufficient detail with regard to specific parameters of processing plasma (*e.g.*, 9-10 ml plasma, 1 ml of 75mM calcium chloride and 2.0 ml of ethanol), similar details regarding parameters specific to the processing of whole blood are conspicuously absent. It is unlikely that a skilled artisan could

extrapolate the same parameters to whole blood given that 40% of the volume of whole blood is cellular. Moreover, given the state of the art at the time the Coelho invention was made *(see* record of instant application), it is unlikely the skilled artisan would have sought to use whole blood in contradiction of the clinically accepted method for obtaining thrombin. There is nothing in the literature to suggest that the use of whole blood without plasma isolation was ever contemplated.

Rock does not remedy the deficiencies of Coelho because Rock describes a method for the stabilization of Factor VIII activity in whole blood or blood plasma. Following the observation that the stability of Factor VIII was adversely effected by collection of blood with anticoagulants that chelated blood calcium, Rock developed a method for blood collection that includes mixing the blood (following collection of the blood with a chelating anticoagulant) with a calcium-heparin solution, whereby calcium is restored to physiologic levels in the presence of a non-chelating anticoagulant (heparin). Rock does not relate to the precipitation of either whole blood or plasma for the recovery of a coagulant material like thrombin.

The Office Action rejects Applicants prior arguments, contending that "Rock was cited in the obviousness rejection to demonstrate that the anticoagulants claimed by Applicant are well known in the art and that their presence in the method of Coelho would have been obvious if not inherent." (see page 17 of Office Action). Applicants respectfully highlight that the present invention is directed to a method for the production of thrombin (a coagulant) and that conventional Ca^{++}-chelating anticoagulants are used in the present invention, but not relevant to the method's novelty, as suggested by the Office Action. Applicants respectfully affirm that neither Coelho nor Rock teach or suggest that a coagulant, for example thrombin, can be extracted from whole blood by ethanol precipitation of the whole blood without the intermediate plasma isolation step.

None of the references cited herein teach or fairly suggest that a coagulant, for example, thrombin can be extracted from whole blood by precipitation of the whole blood without the intermediate plasma isolation step. The ultimate solution of a previously intractable problem can indeed appear to become apparent in hindsight after the final successful step is taken.

However, Applicants respectfully highlight that one cannot use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecate the claimed invention. In re Fritch, 972 F.2d 1260 (Fed. Cir. 1992). Using the inventor's success as evidence that one of ordinary skill in the art would have reasonable expected success represents an impermissible use of hindsight. Life Technologies, Inc. v. Clontech Laboratories, Inc. 224 F.3d 1320 (Fed. Cir. 2000). It is impermissible to engage in a hindsight reconstruction of the claimed invention by using the applicant's structure as a template and selecting elements from references to fill in the gaps. In re Gorman, 933 F.2d 892 (Fed. Cir. 1991).

Applicants respectfully argue that the combination of Coelho and Rock does not render the instant claims as obvious, as the combination fails to support, *inter alia*, any of the seven rationales outlined in the MPEP for determining obviousness *(see* supra and MPEP 2143).

C. OBVIOUSNESS AND INHERENCY

In the context of rejecting claims 1-3, and 7-15 under 35 U.S.C. §103(a) as being unpatentable over Gray in view of Cochrum, and rejecting claims 1-4, 7-18 and 21-22 under 35 U.S.C. §103(a) as being unpatentable over Coelho in view of Rock, the Office Action is dismissive of the new limitations added to claim 1. In particular, the Office Action contends that such limitations are descriptions of the thrombin product using the obvious steps of the instant application and are therefore the inherent result of following those steps. While providing no reasoning or explanation, the Office Action also contends that the purification and optimization of the thrombin product would have been obvious.

In response, with respect to inherency, Applicants respectfully first point out that the principle of inherency has no place in the determination of obviousness under 35 USC Section 103. As pointed out in *In re Spormann,* "inherency of an advantage and it's obviousness are entirely different questions. That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is not known." *In re Spormann,* 150 USPQ 449, 452 (CCPA 1966).

Applicants respectfully point out that claim 1, as amended above, requires that the resulting autologous thrombin contain 80-90% of prothrombin-thrombin proteins, no detectable fibrinogen and 20-30% of baseline levels of anti-thrombin III (ATM). Neither Gray, Cochrum, Coelho or Rock teach such a product, nor can one claim that the autologous thrombin of the prior art inherently meets these criteria given the lack of specifics provided by the cited prior art for preparation of autologous thrombin using whole blood.

Even if one were to ignore the distinction made in the law between inherency and obviousness, contrary to the examiner's assertion, the properties of the final product is not simply an inherent property of practicing the method. Rather, the method practiced will determine the properties of the product. For example, one of skill in the art would have recognized that as part of the steps used in the method of producing thrombin, the precipitation of an anticoagulated whole blood preparation would result in a preparation containing significant levels of cell debris and cellular proteins not present in a similarly processed plasma preparation and therefore, would likely require different handling from plasma. As such, a PHOSITA would not even be motivated to use whole blood in the first place, given its associated problems.

In sum, Applicants respectfully first point out that the principle of inherency has no place in the determination of obviousness under 35 USC Section 103, and that even if one where to ignore this law, the method of the present invention for producing thrombin is not obvious or inherent. Accordingly, at least for this additional reason, Applicants respectfully request that the rejection of claims 1-3, and 7-15 under 35 U.S.C. §103(a) as being unpatentable over Gray in view of Cochrum, and claims 1-4, 7-18 and 21-22 under 35 U.S.C. §103(a) as being unpatentable over Coelho in view of Rock be withdrawn.

D. COELHO, ROCK & SATO

Claims 4-5 remain rejected under 35 U.S.C. § 103(a) as being unpatentable over Coelho *et al.* (U.S. Patent No. 6,472,162; hereinafter "Coelho.") in view of Rock (U.S. Patent No. 4,359,463; hereinafter "Rock") and Sato et al. (U.S. Patent No. 4,812,310; hereinafter "Sato."). In particular, the Examiner adds Sato for the proposition that it teaches that by adding mannitol

to blood, the swelling of blood cells can be prevented during the preservation.

In response, Applicants respectfully point out that Coelho and Rock have been discussed *supra.* No combination of these references teaches the present invention, *inter alia,* because the primary reference used in the rejection (Coelho) has deficiencies which are not remedied by Rock and/or Sato. Applicants respectfully argue that the combination of Coelho, Rock and Sato does not render the instant claims as obvious because, *inter alia,* the combination fails to support any of the rationales outlined in MPEP 2143 *(see* above on p. 11). A PHOSITA would not have recognized that the results of the combination were predictable, and indeed there would have been no reasonable expectation of success to arrive at the present invention by combining the teachings of Coelho, Rock and Sato, as outlined *supra.*

Sato describes the benefit of reduced hemolysis by adding glycerin and mannitol to a blood preservation solution. Sato and Rock fail to remedy the shortcomings of Coelho because, *inter alia,* Sato and Rock relate to anticoagulant preparations commonly used in the art, but not to mixing of whole blood with a precipitating agent to obtain a supernatant containing a coagulant (namely thrombin). None of the references cited herein either alone or in combination teach or fairly suggest that a coagulant, for example, thrombin can be extracted from whole blood by precipitation of the whole blood without the intermediate plasma isolation step.

III. DEFERENCE TO EVIDENCE ON RECORD

On the topic of what level of deference should an examiner give to a declaration submitted to overcome a rejection during prosecution, Applicants respectfully are aware that an Examiner is allowed to be unpersuaded by evidence presented by an Applicant. However, evidence, such as in the form of a § 1.132 declaration, must nonetheless be given proper consideration. For example, BPAI has consistently reversed decisions based on Examiner failing to properly consider the submitted evidence. For example, in Ex parte Malone (Appeal 2009-003894), the BPAI scolded the Examiner for a "largely dismissive" response to the Applicants § 1.132 declaration which presented evidence of non-obviousness. The Board then reversed the obviousness rejection "since the Examiner did not properly consider the submitted

evidence."

Patentability determinations are based on a preponderance of the evidence. "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument." In re Oetiker, 977 F.2d 1443, 1445 (Fed. Cir. 1992) ("[T]he examiner bears the initial burden, on review of the prior art or on any other ground, of presenting a *prima facie* case of unpatentability.").

As stated by the Supreme Court in KSR Intl. Co. v. Teleflex Inc., 550 U.S. 398, 418-19 (2007):

> [A] patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art.

It is incumbent upon the Examiner to identify a reason that would have prompted one of ordinary skill in the art to combine the elements to arrive at the claimed subject matter. *Id.* In re Hedges, 783 F.2d 1038, 1039 (Fed. Cir. 1986) ("If a prima facie case is made in the first instance, and if the applicant comes forward with reasonable rebuttal, whether buttressed by experiment, prior art references, or argument, the entire merits of the matter are to be reweighed").

Here, for example, the Office Action is largely dismissive in response to the Applicants § 1.132 declaration which presented evidence of non-obviousness. In particular, the Office Action contends that the § 1.132 declaration of Dr. Kevy declaration filed 06/14/2007 is insufficient to overcome the rejection of claims 1-18 and 21 based upon the cited references because the declaration "include(s) statements which amount to an affirmation that the affiant has never seen the claimed subject matter before." *(see* Office Action, page 16). First, it stands noted that the Office Action provides no other basis for ignoring the declaration and even on the issue raised, it fails to identify which numbered statement in the § 1.132 declaration of Dr. Kevy are deemed problematic (there are 17 numbered statements/paragraphs). Applicants respectfully indicate

that the declaration in question discusses in detail the present invention in the context of the prior art.

Further, there is no mention in the latest Office Action of paragraphs 8-10 of the § 1.132 declaration of Dr. Mandle, filed January 29, 2010. Although Applicants acknowledge that some of the paragraphs are deemed moot since the McGinnis reference has been overcome, paragraphs 8-10 of Dr. Mandle's declaration, for example, do not relate to McGinnis and instead provide further evidentiary support in the form of another expert's declaration of the state of the art at the time of the invention that establishes the non-obviousness of the present invention. Applicants view the positions taken by the Office in the extensive prosecution history of the present case to be at times conclusory and largely dismissive of the evidence of record.

IV. CONCLUSION

There is no teaching in the cited references or anywhere in the prior art at the time of the present invention which would lead one of skill in the art to conclude that the preparation of autologous thrombin that is ready for use as an anticoagulant from whole blood without an intervening plasma isolation step was obvious. In addition to pointing out the deficiencies in the prior art references cited in the Office Action, Applicants have provided ample evidence in the form of Declarations under 37 C.F.R. 1.132, references from the pertinent literature and product information with respect to the thrombin processing device described in ThermoGenesis U.S. patent no. 6,472,162 in support of the position that the skilled artisan would not have been motivated to remove the plasma isolation step from the method of making thrombin from autologous blood.

There being no other outstanding issues, it is believed that the application is in condition for allowance, and such action is respectfully requested. Should the Examiner believe that anything further is desirable in order to place the application in better condition for allowance, the Examiner is invited to contact Applicants' undersigned attorney at the telephone number listed below.

The undersigned hereby authorizes the Commissioner to charge any fee insufficiency and credit any overpayment associated with this submission to Deposit Account No. 08-1935.

Respectfully submitted,

/s/ *Shahrokh Falati*

Date: August 25, 2010

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Attorney for Applicant(s)
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Encls.
(1) ThermoGenesis Corp. (owner of Coelho patent) article (JECT 39:18-23, 2007);
(2) *Blood components* and *Blood Component Production* (Cornell University, College of Veterinary Medicine);
(3) *What is Cryoprecipitated AHF?*
(4) *Interference of blood cell lysis on routine coagulation testing (*Arch. Pathol. Lab Med. 2006; 130: 181-184)

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RESPONSE TO OFFICE ACTION DATED APRIL 26, 2010

SERIAL NO. 10/765,694

ENCLOSURE 1

JECT 2007;39:18-23
The Journal of The American Society of Extra-Corporeal Technology

Whole Blood Thrombin: Development of a Process for Intra-Operative Production of Human Thrombin

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Abstract: Thrombin-based clotting agents currently used for topical hemostasis with absorbable sponges, fibrin sealants, and platelet gels are primarily derived from bovine or pooled human plasma sources. Autologous thrombin has important safety advantages in that it does not carry the same safety concerns as pooled plasma-derived products and it avoids exposure to risks associated with bovine-derived proteins. The goal of our research was to develop a rapid, reliable, and simple to perform process to generate autologous human thrombin in the intra-operative setting, from patient whole blood as the starting source material. Using whole blood instead of plasma as the starting material, it is possible to avoid the inherent delay in thrombin availability associated with a primary step of plasma isolation. In this study, we varied several key processing parameters to maximize thrombin production, reproducibility and stability. Autologous thrombin production was generated using a dedicated, single use disposable with a sterile reagent. The disposable consists of a tubular reaction chamber containing glass microsphere beads to activate the alternative pathway of the coagulation cascade. At the end of the process, thrombin-activated serum was harvested from the reaction chamber. The average activity of the thrombin produced at room temperature by this system was 82.8 ± 15.9 IU/mL. The total processing time was <30 minutes. The system was compatible with Anticoagulant Citrate Dextrose-Solution A (ACD-A) (8%–12%). The average volume of thrombin harvested from each aliquot of blood was 7.0 ± 0.3 mL, and the stability of thrombin was observed to be temperature dependent, with cold storage better preserving thrombin activity. Clot times with platelet concentrates at 1:4.3 and 1:11 ratios (thrombin to platelet concentrate) were <10 and 20 seconds, respectively. A process for the preparation of thrombin from whole blood, under conditions compatible with the resources of an operating room, has been developed. The device is simple to use, requires 30 minutes, and can consistently produce thrombin solutions that achieve rapid clotting of platelet concentrates, plasma, and fibrinogen concentrates even when mixed at thrombin to blood product ratios of 1:11. **Keywords:** blood, human thrombin, stability, fibrinogen, clot. *JECT. 2007;39:18–23*

Thrombin (activated factor II) is a coagulation protein that has many effects in the coagulation cascade. It is a serine protease that converts soluble fibrinogen to an active form that assembles into fibrin (1). Thrombin also activates factor V, factor VIII, factor XI, and factor XIII. This positive feedback accelerates the production of thrombin. Factor XIIIa is a transglutaminase that catalyzes the formation of covalent bonds between lysine and glutamine residues in fibrin. The covalent bonds increase the stability of the fibrin clot.

Thrombin has been widely used in various surgical procedures for the reduction of bleeding in combination with collagen sponges (2–5). A combination of thrombin with a concentrated fibrinogen source (i.e., single donor cryoprecipitate or purified fibrinogen from pooled plasma) is also commonly used in clinical practices as an adjunct to achieve hemostasis (6). In addition to its activity in the coagulation cascade, thrombin also promotes platelet activation through activation of protease-activated receptors on the platelet. This activation pathway and clot formation has led to the use of thrombin in combination with platelet concentrates to form platelet gels that are being used in orthopedic, oral, and maxillofacial surgery as a means to enhance wound healing. It is proposed that bone growth can be enhanced because of increased level of tissue growth factors associated with platelet granules (7–10).

The choice for the source material to derive thrombin used in surgical procedures is extremely important for the resulting thrombin risk profile to the patient. The safest thrombin source is a patient's own blood. However, the majority of thrombin used today is of bovine origin. The immunologic response elicited by bovine thrombin prepa-

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rations and their clinical sequela are well established. Because bovine thrombin preparations are not completely pure, an immunologic response can be elicited by other bovine plasma contaminants such as factor V. The bovine-elicited antibodies have the potential to cross-react with their corresponding human factors. For example, some of the most serious adverse effects are mediated by antibodies with cross-reactivity to human factor V. These factor V reactive antibodies can cause a range of coagulopathy-associated symptoms that may lead to adverse reactions, including severe and life-threatening bleeding (11,12). Another concern particularly for thrombin sourcing from bovine and pooled human products is possible exposure to prions causing variant Creutzfeldt-Jacob disease (vCJD) in humans (13). Thus, the most preferred source material for preparing thrombin used in surgical procedures is autologous thrombin.

Until recently, most of the commercially available thrombin preparations were derived from large pools of mixed donor plasma by Cohn-Oncley fractionation (14). Active thrombin was produced from purified prothrombin using several different methods including use of different coagulation proteins (15,16) or snake venom as activators (17–20). Although these methods are well adapted for industrial scale production of thrombin from large pools of plasma, they are not practical methods for the preparation of single donor autologous thrombin.

We have previously reported the development of a simple and reliable method for development of autologous thrombin from single donor plasma (21). Thrombin production can be readily initiated by adding an excess of calcium ions to citrated plasma, which allows initiation of the clotting cascade and finally to the production of thrombin. However, the use of thrombin prepared in this simple manner is of limited value because of the slow rate of thrombin production and the low concentration of thrombin produced. Our method overcomes these limitations by using glass beads to provide a negatively charged surface to greatly accelerate the rate of thrombin production. Furthermore, the use of the thrombin reagent allows the stabilization of the thrombin by blocking the inhibitory actions of protein C, protein S, and particularly, antithrombin III (1). The resulting thrombin is sufficiently stable that it can be stored for hours after its production and still maintain clinical use.

In this study, we have developed a reliable technique to generate autologous human thrombin in the intraoperative setting from whole blood instead of plasma as the starting source material within a 30-minute period. With this method, it is possible to avoid a delay in thrombin availability to the surgical team caused by elimination of a plasma separation step and requires only 12 mL of whole blood. The goal of this research was to optimize the thrombin production process using whole blood and to characterize the thrombin produced for activity and stability. The performance characteristics of the thrombin were evaluated both quantitatively by measuring thrombin activity using a functional clot assay and by measuring the ability and time required for the thrombin to complete gel formation with platelet-rich plasma (PRP).

MATERIALS AND METHODS

Collection of Anticoagulant Citrate Dextrose-Solution A Blood

Fresh blood was obtained from our in-house donation program. The blood was collected in 60-mL Anticoagulant Citrate Dextrose-Solution A (ACD-A) (8%) anticoagulated syringes obtained from pre-screened healthy volunteers. The blood was handled according to established procedures for the collection and processing of human blood products. Each blood unit was processed within 6 hours of initiation of the collection.

Blood Thrombin Processing System

The blood thrombin processing system (BTPS) consists of two parts: a tubular reaction chamber containing negative surface charge beads needed for initiation of thrombin formation and a reagent consisting of calcium chloride and ethanol at concentrations of 25 mmol/L and 66%, respectively.

Preparation of Thrombin

Twelve milliliters of blood and 4 mL thrombin reagent were added to the reaction chamber. The contents were mixed and incubated for 15 minutes. At the end of the 15-minute incubation period, the blood/beads were mixed by agitation to break any formed clots, followed by a centrifugation step for 12 minutes at 2100*g*. At the end of the centrifugation step, thrombin activated serum (top fluid) was harvested from the reaction chamber.

To establish intra-variability of the thrombin production system, the same blood was used with 10 different thrombin-producing systems. Inter-variability was assessed by determining the thrombin activity in 20 thrombin-producing systems from different blood units. To study the influence of ambient temperature on thrombin production, the process was performed at two different temperatures: 17°C and 25°C.

Preparation of PRP

PRP was prepared using 60-mL ACD-A anticoagulated blood using the Magellan device as per the manufacturer's instructions.

Thrombin Activity and Quantitative Assessment of Thrombin

Thrombin activity was analyzed using the modified Clauss method (22) as described previously (21). Briefly,

200 μL of a 2.0-mg/mL pre-warmed (37°C) solution of fibrinogen (catalog F-4883; Sigma, St. Louis, MO) was added to 100 μL of the thrombin sample. The time required for clot formation was recorded using a fibrometer (Fibro System; Becton-Dickinson, Franklin Lakes, NJ). The thrombin activity in the samples was determined by correlating the time to clot formation to a standard curve generated with titrations of thrombin (catalog 50502; Biopool US, Ventura, CA). Because each time-point on the standard curve corresponds to the thrombin activity needed to clot the standard concentration of fibrinogen, the thrombin activity in an unknown sample can be extrapolated from the time to clot formation.

Clot Time and Qualitative Assessment of Thrombin

Clot times were assessed by mixing PRP with variable concentrations of thrombin. Thrombin preparations of different activity used for this test were prepared by serial dilutions of the starting thrombin with saline solution. One part of thrombin and 3.3 or 10 parts of PRP were mixed in a cup, and the time to clot formation was assessed. To establish the relationship between clot time and thrombin activity, both analyses were performed, and a correlation was established.

Assessment of Stability

The stability of the produced thrombin was assessed by storing 3 mL of thrombin either at the production temperature (i.e., 17°C or 25°C) or at 4°C. Thrombin activity was assessed at time 0 (after preparation) and after 2 and 4 hours of storage.

Statistical Analysis

All results are presented as mean and SD. Coefficient of variation (CV) was calculated for intra- and inter-variability. Significance between groups was assessed using the Student *t* test.

RESULTS

Intra–Reproducibility of Thrombin Produced at 25°C

To establish the intra-variability of the thrombin production system, the same blood was used with 10 different thrombin-producing systems. Using blood from the same unit and 10 different BTPSs (intra-reproducibility), the thrombin activity was 82.8 ± 15.9 IU/mL, with a CV of 21% (Table 1).

Inter-Reproducibility of Thrombin Produced at 25°C

Inter-variability was assessed by determining the thrombin activity in 20 thrombin-producing systems from different blood units. The inter-reproducibility was established from 10 different blood units, using BTPSs from the same lot of material. Thrombin activity was found to be 67.9 ± 19 IU/mL (range, 47–117 IU/mL), with a CV of 32% (Table 2).

Table 1. Thrombin production at 24°C: intra-reproducibility of thrombin produced from whole blood using the BTP system.

	Thrombin Harvest Volume (mL)	Thrombin Activity (IU/mL)
Mean ± SD (n = 10)	7.0 ± 0.3	82.8 ± 15.2
CV	4%	21%
Range (min – max)	6–7	59–104

Thrombin activity is expressed as mean ± SD.

Short-Term Stability of Thrombin Produced at 25°C

The results are shown in Table 3. For thrombin prepared at 25°C, the activity was 67.9 ± 19.0 IU/mL, with a CV of 32%. When samples were stored at 4°C for 4 hours, the thrombin activity was 59.8 ± 15.9 IU/mL, with a CV of 31%. The data also showed that thrombin prepared at normal ambient temperature (25°C) is sufficiently stable to effectively form fibrin clots and platelet gels when stored up to 4 hours at 4°C.

Correlation Between Thrombin Activity and Clot Time With PRP

Thrombin activity and clot times were analyzed in 83 samples. Clot formation was analyzed by mixing 1 part of thrombin and 3.3 or 10 parts of PRP. Results for thrombin activity and in vitro clot times with PRP (1 part of thrombin + 3.3 parts of PRP) using different blood units are shown in Figure 1. Data from this study show that acceptable clot times (≤15 seconds) with PRP can be achieved by mixing 1 part of thrombin + 3.3 parts of PRP. Results suggest that a minimum thrombin activity of 15–20 IU/mL will be needed to achieve acceptable clot times. There was no significant difference in clot times between units with a thrombin activity of 30–45 IU/mL compared with units with a thrombin activity of 46–80 IU/mL (8.2 ± 1.2 and 7.7 ± 1.5 seconds, respectively; no significant difference). Units with a thrombin activity of 0–30 IU/mL had significantly longer clot times compared with units with a thrombin activity of 30–45 IU/mL (15.0 ± 6.4 and 8.2 ± 1.4 seconds, respectively; $p < .0001$). The total population (n = 64) with a thrombin activity of >30 IU/mL had 7.9 ± 1.5

Table 2. Thrombin production at 24°C: inter-reproducibility of thrombin produced from whole blood using the BTP system.

	Thrombin Harvest Volume (mL)	Thrombin Activity (IU/mL)
Mean ± SD (n = 20)	7 ± 0.4	67.9 ± 19.0
CV	7%	28%
Range (min – max)	6–7	47–117

Thrombin activity is expressed as mean ± SD.

Table 3. Short-term stability of thrombin produced at 24°C from whole blood stored at 4°C.

	Thrombin Activity (IU/mL)	
	T_0	T_4 on Ice
Mean ± SD (n = 20)	67.9 ± 19.0	59.8 ± 15.9
CV	32%	31%
Range (min – max)	47–117	45–102

Thrombin activity is expressed as mean ± SD.

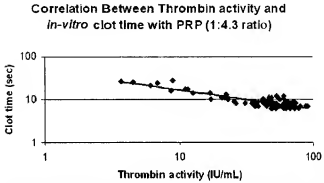

Figure 1. Correlation between thrombin activity and in vitro clot times with PRP (1 part of thrombin + 3.3 parts of PRP) using different blood units.

seconds in clot time. Data from this experiment suggest that clot times with platelets concentrates at 1:4.3 ratios were <10 seconds.

Results for correlation between thrombin activity and in vitro clot times with PRP (1 part of thrombin + 10 parts of PRP) using different blood units are shown in Figure 2. Units with a thrombin activity of 0–30 IU/mL had significantly longer clot times compared with units with a thrombin activity of 30–45 IU/mL (23.1 ± 4.8 and 14.2 ± 2.6, respectively; $p < .0001$). The total population (n = 45) with a thrombin activity of >30 IU/mL had 12.3 ± 1.8 seconds in clot time. Results suggest that a minimum thrombin activity of 30 IU/mL will be needed to achieve acceptable clot times (≤15 seconds).

Quality of BTPS–Thrombin Produced at Different Temperatures

The ambient temperature in the operating room, where the whole blood thrombin would be produced, can vary; hence, we assessed the quality of thrombin at 17°C and 25°C (corresponding to 63°F and 77°F). Within 10 minutes of production, there was significant difference in the thrombin activity between thrombin produced at 17°C and 25°C (66 ± 20 and 77 ± 30 IU/mL, respectively; $p > .05$).

Stability of BTPS-Produced Thrombin

The stability of the BTPS-produced thrombin was assessed by measuring thrombin activity at 2 and 4 hours

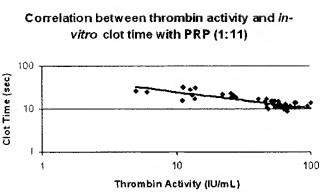

Figure 2. Correlation between thrombin activity and in vitro clot times with PRP (1 part of thrombin + 10 parts of PRP) using different blood units.

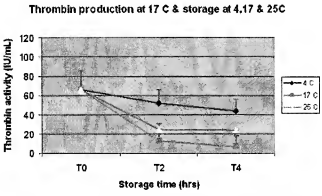

Figure 3. Stability of thrombin produced using the BTPS. The thrombin was produced at 17°C and stored for 2 and 4 hours at different temperatures (4°C, 17°C, and 25°C). Thrombin activity was analyzed by the method as described in the Materials and Methods.

after production. The stability was determined at the production temperature (17°C and 25°C, respectively) and at 4°C.

Stability of BTPS–Thrombin Produced at 17°C

Thrombin produced at a lower ambient temperature (17°C) did not retain its thrombin activity as well as thrombin produced at higher ambient temperatures. After 4 hours of storage at 4°C, the thrombin activity had decreased to 44 ± 11.0 IU/mL. After 4 hours of storage at 17°C and 25°C, we found that the thrombin activity decreased to 8 ± 0.1 and 24.2 ± 12.7 IU/mL, respectively (Figure 3).

Stability of BTPS–Thrombin Produced at 25°C

When thrombin was prepared at normal ambient temperature (25°C) and stored at that temperature, the thrombin activity declined to 47.2 ± 25.2 IU/mL after 4 hours (Figure 4). However, when the same thrombin was stored at 4°C, the decrease in thrombin activity was not as pronounced as when stored at 17°C (74.8 ± 26.9 and 36.8 ± 21.2 IU/mL, respectively) after 4 hours. The data suggest

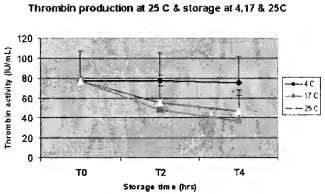

Figure 4. Stability of thrombin produced using the BTPS. The thrombin was produced at 25°C and stored for 2 and 4 hours at different temperatures (4°C, 17°C, and 25°C).Thrombin activity was analyzed by the method as described in the Materials and Methods.

that thrombin prepared at normal ambient temperature (25°C) can be stored for up to 4 hours at 4°C and still maintain its effectiveness.

DISCUSSION

The results of this study indicate that a practical process for the routine production of human thrombin from autologous whole blood in the intra-operative setting can be achieved. The whole blood technique we developed is derived from the method that we previously commercialized using single donor plasma. Thrombin used in surgical procedures as an adjunct to hemostasis (3–10) is primarily derived from bovine sources and is associated with major concerns about adverse reactions, e.g., antibody formation against human FV leading to bleeding episodes (11,12) and transmission of bovine prions possibly causing vCJD (13). Use of autologous thrombin is an attractive alternative choice in surgeries because it avoids these infectious disease and immunogenicity risks. This whole blood procedure provides a faster method than our previous plasma procedure because it eliminates the need for a centrifugation and harvest step for first collecting the plasma. The entire procedure can readily be performed in ~30 minutes. In this study, we evaluated the stability of thrombin produced from human whole blood. The blood we used was fresh blood collected from normal donors and tested within 8 hours of collection to replicate intended use conditions. In surgery, the blood will be taken directly from the patient, and thrombin will be produced intra-operatively.

The enzymatic activity of thrombin to be used either by itself or in combination with fibrinogen or platelets is important for the speed of clot formation (23). Typically for most applications, bovine thrombin is diluted to 500 and 1000 IU/mL. The thrombin produced in this study ranged in activity from 59 to 104 IU/mL, with a mean of 83 IU/mL. This thrombin activity is very similar to our previous observation with plasma using a thrombin processing device (TPD) (21). In addition, we have previously shown that TPD-produced thrombin can activate platelets to release α-granule content, and consequently, growth factors, comparably with commercially available thrombin (24).

When 10 units of BTPS–thrombin were produced from the same unit of blood, the CV was 21%, indicating the ability of the BTPS to produce thrombin with consistent thrombin activity. Furthermore, when blood from different donors was used, the CV increased to 28%. Thus, there is variability between donors/blood in the ability to produce active thrombin using the BTPS. This would be expected because availability of prothrombin and other coagulation factors needed for formation of the prothrombinase complex can vary between donor blood. The results suggest that the BTPS is able to produce relatively consistent thrombin with acceptable activity.

Using in vitro clot time with PRP as a qualitative measure of the efficacy of the thrombin, we found that the clot time depends on the ratio of thrombin to PRP. In most cases, a clot time of 10–15 seconds was equal to instant clotting, because of the manual variability of the test. Data from this study showed that clot times with platelet concentrates at 1:4.3 and 1:11 ratios were <10 and 20 seconds, respectively. This in vitro clot time suggests that lower levels of thrombin activity than what is commonly used today would be sufficient for adequate clot formation in vivo. In fact, using a combination of thrombin and fibrinogen in a fibrin sealant, it has been shown that a thrombin with an activity of 50–100 IU/mL had a higher adhesiveness compared to one with a thrombin activity of 500 IU/mL (25).

Typically enzymes are sensitive to temperature, and the rate of activation and degradation is dependent on the specific optimal temperature for each enzyme. Our data suggested that thrombin produced at 24°C had a higher activity and retained its activity for a longer time compared with thrombin produced at a lower temperature (17°C). This observation is very important for the user. Because thrombin is an active enzyme, the user has to be aware of both the production temperature (the ambient temperature in the room) and the storage temperature for optimal efficacy of the product. For example, if the ambient temperature is 25°C, the BTPS–thrombin can be stored for up to 2 hours without any special precautions, i.e., it can be stored on the bench. If longer storage time is needed, the thrombin should be stored at 4°C. However, if the ambient temperature is in the 16–18°C range, as is the case in certain surgical procedures, the shelf-life of the thrombin is shorter, and it should be used within 1–2 hours of production or stored on ice.

In conclusion, we have demonstrated that it is practical to produce active and effective thrombin from whole

blood at low and normal ambient temperatures. We have also showed that the activity and in vitro clot times of thrombin are dependent on both the production and storage temperatures. Thrombin produced at 17°C and 25°C retained clotting efficacy for 4 hours when stored at 4°C. Thus, we have developed a simple, rapid, and reliable method for generation of stable thrombin from the patients' own blood in ~30 minutes.

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ENCLOSURE 2

Blood components

Whole blood is collected from a donor animal for blood transfusion purposes into a blood bag containing citrate phosphate dextrose as the anticoagulant. Donor animals should be selected with care and strict attention should be paid to the blood collection technique to maintain sterility at all times. Once the blood has been collected, it can be kept and used in its natural state or can be converted into a variety of components. These blood components are packed red blood cells, platelet-rich plasma, platelet concentrates, fresh plasma, fresh frozen plasma, frozen plasma, cryoprecipitate and cryosupernant. Double, triple and quadruple blood bags (a single whole blood collection bag with various satellite bags) are used for producing and separating components within a sterile closed environment. Blood component production is highly desirable for several reasons, including maximization of the yield of products from a single blood donation and ability to use the optimal products (in high concentrations) for specific diseases (thus minimizing the amount of unnecessary foreign material the recipient animal is exposed to). The most commonly available blood components are whole blood, packed red blood cells, fresh frozen plasma and cryoprecipitate.

Some definitions may be useful for understanding blood components:

Whole blood: This is blood collected directly from a donor animal into a blood transfusion bag containing citrate-phosphate-dextrose with (CPDA-1) or without adenine (CPD) as an anticoagulant. In general, for dogs, a 500 ml transfusion bag is used (which contains approximately 63 ml of anticoagulant, obtaining approximately 450 ml blood from the donor dog).
Whole blood can be used immediately or stored at 4 C for future transfusion or separation into blood components. Additives can be used to improve red cell storage viability (see below).

Packed red blood cells: These are a concentrated source of red blood cells that remain in a small amount of plasma upon removal of supernatant plasma after centrifugation of the blood transfusion bag containing whole blood.
Packed cells are an excellent source of red blood cells for anemic animals that need the additional oxygen-carrying capacity these cells provide.
Packed red cells can be used immediately or stored at 4 C. They need dilution in sterile isotonic solution prior to infusion (due to the high hematocrit, packed cells without dilution are very thick and flow sluggishly through infusion lines). The current standard for red cell storage is that there should be about 75% posttransfusion viability after storage, that is at least 75% of the transfused red cells must remain in the recipient's circulation 24 hours after transfusion. The posttransfusion viability of canine red cells collected into CPDA-1 is 20 days.

Additives have been developed that can be added in a sterile fashion

(they are attached to the original transfusion bag as a satellite bag) to packed red cells (or whole blood bags) to optimize red cell storage viability. These additives usually contain dextrose and adenine for red cell energy metabolism and mannitol, which decreases red cell lysis. Several additives have been evaluated with canine red blood cells. Both Nutricel and Adsol increase the posttransfusion viability of stored canine red cells to 35 and 37 days, respectively.

Platelet-rich plasma (PRP): Platelet-rich plasma is produced by separating plasma from red blood cells (within 6 hours of whole blood collection) using a slow spin to prevent pelleting of the platelets. Platelet-rich plasma is useful for the treatment of disorders of platelet number and function but must be infused within 8 to 12 hours (maintained at room temperature) due to platelet instability (which limits its use).

Platelet concentrates: Platelet concentrates are produced by separating most of the plasma from platelet-rich plasma.
Platelet concentrates are used similarly to platelet-rich plasma.

Platelet-poor plasma (PPP): Platelet-poor plasma is produced by separating plasma from red blood cells using a high spin to pellet platelets with the red cells. This is the starting point for production of most blood components. Separation of platelets is desirable because they provide an additional source of foreign antigens and micro-aggregation can cause transfusion reactions. Approximately 200 to 400 ml of plasma are obtained from each whole blood transfusion bag.
One unit of plasma is defined as that obtained from a single whole blood transfusion bag.

Fresh plasma: This is platelet-poor plasma that is separated from red cells and infused **within 6 hours** of blood collection (with the blood bag being maintained at 4 C until separation).

Fresh frozen plasma (FFP): This is platelet-poor plasma that is separated from red cells **within 6 hours** of blood collection and frozen in a dedicated freezer (at or below -20 C). Fresh frozen plasma and fresh plasma contain all coagulation factors and plasma proteins (such as albumin).
Fresh frozen plasma is stable for 1 year if maintained in a dedicated freezer (one that does not undergo freeze-thaw cycles like household freezers) at or below -20 C.
One unit of FFP is defined as that obtained from a single whole blood transfusion bag (approximately 250 ml).

Cryoprecipitate (CPP): Cryoprecipitate is a concentrated source of von Willebrand factor, fibrinogen, factor VIII and fibronectin. It is produced by slow-thawing (at 4 C) fresh frozen plasma, followed by centrifugation at 4 C. Cryoprecipitable proteins (those mentioned above) precipitate at this temperature and are maintained in a very small amount of remaining plasma (approximately 1/10 of the starting volume of FFP, or about 20 to 30 ml).
Cryoprecipitate is stable for 1 year from the date of collection of the whole blood for transfusion purposes (not the date of preparation of the product) if maintained at or below -20 C in a dedicated freezer.
One unit of cryoprecipitate is defined as that obtained from a single FFP bag (approximately 250 mL plasma).

Cryosupernant (Cryosuper): Cryosupernant is the remaining plasma after removal of the pelleted cryoprecipitate. This is a source of all coagulation and plasma proteins, except for factor VIII, fibrinogen, von Willebrand factor and fibronectin.
Cryosupernant is stable for 5 years if stored in a dedicated freezer at or below -20 C.

Frozen plasma: Frozen plasma has several sources. It can be plasma that is separated and frozen **longer than 6 hours** after whole blood collection. It can be fresh plasma that is not used within 6 hours of collection and then frozen. It can be fresh frozen plasma maintained for **longer than 1 year** in a dedicated freezer. Frozen plasma lacks certain coagulation factors which are quite unstable, including factor VIII, von Willebrand factor and factor V.

A schematic illustration of how these major blood component products are produced may be helpful.

ENCLOSURE 3

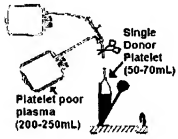
What is Cryoprecipitated AHF?

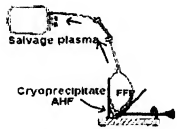
Cryoprecipitated AHF (Cryoprecipitated Antihemophilic Factor or CRYO) is the cold-insoluble portion of plasma that precipitates when FFP has been thawed between 1-6C.

CRYO contains:

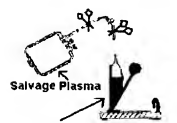
- Factor XIII
- Fibronectin
- Fibrinogen
- Factor VIII:vWF (von Willebrand Factor)
- Factor VIII:C

How to Make Cryoprecipitate:





Fresh Frozen Plasma spun down



1. Platelet poor plasma from CPDA-1 or CPD whole blood unit that has been frozen to make FFP is allowed to thaw in a refrigerator at 1-6C overnight or about 8-10 hours until the plasma has a slushy consistency. Technically, This is when approximately one tenth of the contents is still frozen.
2. Centrifuge the plasma at 1-6C using a Heavy Spin to separate the plasma from the cryoprecipitate.
3. Place centrifuged, thawed plasma in a plasma expressor and allow the supernatant to flow slowly into a transfer bag. The cryoprecipitate paste will adhere to the side of the bag or to the ice. The cryo will appear to be a small white mass in the original plasma bag.
4. Units of CRYO can be pooled prior to freezing and storage. If pooled promptly after preparation using aseptic technique and refrozen immediately, the resulting component is labeled " Cryoprecipitated AHF pooled" with the number of units pooled stated on the label. The facility preparing the pool must maintain a record of each individual donor traceable to the unique pool number.
5. Leave about 10-15mL of supernatant plasma in the bag for resuspending the cryoprecipitate after thawing.
6. Refreeze the cryoprecipitate immediately. Remember, cryoprecipitate must be refrozen within 1 hour of preparation and store at -18C or cooler.

Cryoprecipitated AHF can be used:

1. For controlling the bleeding associated with fibrinogen deficiency.

2. For treating Factor XIII deficiency.
3. To make **"Fibrin Glue"**, a substance composed of cryoprecipitate and topical thrombin. When combined, they produce an adhesive substance that, applied to a surgical site can reduced bleeding. It is also know as the **"Human Glue"**
4. As a second -line therapy for von Willebrand's disease and hemophilia A. Cryoprecipitate should be used only if viral-inactivated Factor VIII concentrates are not available for management of these patients.

Storage and Expiration of CRYO:

- Cryoprecipitate must be stored at -18C or colder, preferably -30C or colder.
- It expires 1 year from the date the of phlebotomy, not from the date the cryo was prepared.
- Once cryoprecipitate is thawed, it must be kept at room temperature (20-24C). Administration must begin as soon as possible or within 6 hours after thawing.

Quality Control for CRYO:

- Cryoprecipitate is prepared from fresh frozen plasma from CPDA-1 or CPD whole blood at any time within the 12 month expiration of the fresh frozen plasma.
- Cryo should contain: minimum of 150 mg of fibrinogen and minimum of 80 IU (International Units) of Factor VIII (AHF).
- In test performed on pooled components, the pool shall contain a minimum of 150 mg of fibrinogen and 80 IU of coagulation Factor VIII times the number of components in the pool.

Other Products Associated with Cryoprecipitated AHF	
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RESPONSE TO OFFICE ACTION DATED APRIL 26, 2010

SERIAL NO. 10/765,694

ENCLOSURE 4

Interference of Blood Cell Lysis on Routine Coagulation Testing

Giuseppe Lippi, MD; Martina Montagnana, MD; Gian Luca Salvagno, MD; Gian Cesare Guidi, MD

• *Context.*—Preanalytical factors influencing the reliability of laboratory testing are commonplace. It is traditionally accepted that hemolytic samples are unsuitable for coagulation assays because of the release of hemoglobin, intracellular components, and thromboplastic substances from damaged blood cells.

Objective.—To evaluate the influence of blood cell lysis on routine coagulation testing.

Design.—Twelve aliquots prepared by serial dilutions of homologous lysated samples collected from 10 different subjects, and displaying a final percentage of lysis ranging from 0% to 9.1%, were tested for prothrombin time, activated partial thromboplastin time, fibrinogen, and dimerized plasmin fragment D. Lysis was achieved by subjecting citrated whole blood to a freeze-thaw cycle.

Outcome Measures.—Interference from blood cell lysis on routine coagulation testing.

Results.—Statistically significant increases in prothrombin time and dimerized plasmin fragment D were observed in samples containing final lysate concentrations of 0.5% and 2.7% respectively, whereas significant decreases were observed in activated partial thromboplastin time and fibrinogen in samples containing a final lysate concentration of 0.9%. The current analytical quality specifications for desirable bias are ±2.0% for prothrombin time, ±2.3% for activated partial thromboplastin time, and ±4.8% for fibrinogen. Percent variations from the baseline values exceeding the current analytical quality specifications for desirable bias were achieved for lysate concentrations of 0.9% (prothrombin time and activated partial thromboplastin time) and 1.8% (fibrinogen), corresponding to average free plasma hemoglobin concentrations of 1.7 and 3.4 g/L, respectively.

Conclusion.—Our results confirm that, although slightly hemolyzed specimens might still be analyzable, a moderate blood cell lysis, as low as 0.9%, influences the reliability of routine coagulation testing. Because the interference in coagulation assays has a wide interindividual bias, we do not recommend lysis correction and we suggest that the most appropriate corrective measure should be free hemoglobin quantification and sample recollection.

(*Arch Pathol Lab Med.* 2006;130:181–184)

Coagulation testing is a central aspect of the diagnostic approach to patients with hemostasis disturbances, and it is pivotal for monitoring antithrombotic therapies with either heparins or oral anticoagulants.[1,2] Among major determinants of coagulation testing, the standardization of the preanalytical phase exerts a major influence on result reliability. A standardized procedure for specimen collection was demonstrated to be essential to achieve accurate and precise measurements, which might finally provide appropriate and suitable clinical information.[3] However, there are additional circumstances besides specimen collection that might influence the results of coagulation testing; these additional circumstances might also generate misleading results and induce an inappropriate diagnostic or therapeutic approach to the patients. Problems arising from a cumbersome blood draw, such as unsatisfactory attempts to draw blood, difficulty locating easy venous accesses, and missing the vein, are anecdotally known to produce major interference in routine coagulation assays, and the use of hemolyzed specimens has been discouraged to avoid unreliable results.[4,5] However, to our knowledge, little is known about the true influence of unsuitable samples caused by blood cell lysis on routine coagulation testing. Therefore, we evaluated the interference of in vitro blood cell lysis on prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and dimerized plasmin fragment D (D-dimer) testing.

MATERIALS AND METHODS

Experiment Design and Blood Sampling

On the morning of the first day of the evaluation, 4.5 mL of blood were separately collected in 2 siliconized vacuum tubes containing 0.5 mL of 0.105M buffered trisodium citrate (Becton Dickinson, Oxford, United Kingdom), using a 20-gauge, 0.80 × 19-mm Venoject multisample straight needle (Terumo Europe NV, Leuven, Belgium) from 10 healthy volunteers. Volunteers gave an explicit informed consent for the investigation. Volunteers were selected on the basis of a homogeneous range of values for white blood cell counts (3.25–4.98 × 10^3 cells/μL), platelet count (153–281 × 10^3 cells/μL), and hemoglobin concentration (137–146 g/L). The first specimen (sample 1) was gently mixed by inverting the tube 4 to 6 times and immediately stored at −70°C,

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whereas the second specimen (sample 2) was gently mixed by inverting the tube 4 to 6 times and then centrifuged at 3000*g* for 10 minutes at 10°C. Plasma was separated and stored at −70°C.

On the morning of the second day of the evaluation, blood was collected into 6 additional siliconized vacuum tubes containing 0.105M buffered trisodium citrate (Becton Dickinson) using a 20-gauge, 0.80 × 19-mm Venoject multisample straight needle (Terumo) from each of the 10 volunteers. All samples were gently mixed by inverting the tube 4 to 6 times, pooled, and divided into 12 aliquots of 2 mL each. Samples 1 and 2 were thawed. Twelve serial dilutions, obtained by mixing samples 1 and 2, were prepared by adding 200 μL of each dilution to the 2-mL aliquots of blood collected on the second day. Final whole blood lysate concentrations in the mixtures of samples ranged from 0% to 9.1%, roughly corresponding to average free plasma hemoglobin concentrations ranging from 0 to 17 g/L, thus, almost representative of the degree of hemolysis that we observe in specimens sent to our laboratory. The blood samples were centrifuged at 3000*g* for 10 minutes at 10°C, and plasma was separated and immediately analyzed.

Laboratory Testing

Hemolysis was assayed by measuring the concentration of free plasma hemoglobin by the reference cyanmethemoglobin method on a UV-1700 Spectrophotometer (Shimadzu Italia S.l.r., Milan, Italy).[8] White blood cell and platelet counts were performed on an ADVIA 120 (Bayer Diagnostics, Newbury, Berkshire, United Kingdom). Routine coagulation measurements were performed on a Behring Coagulation System (Dade-Behring, Marburg, Germany), using proprietary reagents: Thromborel S (lyophilized human placental thromboplastin), Pathromptin SL (vegetable phospholipid with micronized silica), and Multifibren U, for PT, aPTT, and fibrinogen testing, respectively. Because hemolytic samples have a greater than usual inherent spectrophotometric absorbance in conventional clotting assays, PT, aPTT, and fibrinogen measurements were performed at a different wavelength (570 nm), uniquely available on the BCS analyzer. Using this alternative assay configuration, the absorbance threshold for recording the coagulation time is dynamically increased by the evaluation software of the analyzer. The threshold level depends on the inherent absorbance and is automatically calculated by the analytic system. Plasma D-dimer was measured with the Vidas DD, a rapid, quantitative, automated enzyme-linked immunosorbent assay with fluorescent detection, on the Mini Vidas Immunoanalyzer (bioMérieux, Marcy l'Etoile, France). Actual reference ranges were between 10.8 and 13.1 seconds for PT, 26.2 and 36.0 seconds for aPTT, 150 and 400 mg/dL for fibrinogen, and less than 500 ng/mL for D-dimer. All measurements were performed in duplicate within a single analytical session and the results were averaged. Analytical imprecision, expressed in terms of the mean interassay coefficient of variation, was between 2% and 5%, according to the manufacturers.

Statistical Evaluation

Differences between coagulation measurements on aliquots of the same sample containing serial concentrations of homologous lysate were evaluated by paired Student *t* test. Statistical significance was set at $P < .05$. Percentage variations from the baseline value were further compared with the current analytical quality specifications for desirable bias, as derived from the intraindividual and interindividual variations (±2.0% and ±0.24 seconds for PT; ±2.3% and ±0.69 seconds for aPTT, ±4.8% and ±15 mg/dL for fibrinogen, respectively).[9] This comparison was not feasible for D-dimer testing, because no definitive data on its biologic variability in healthy individuals are available, to our knowledge.

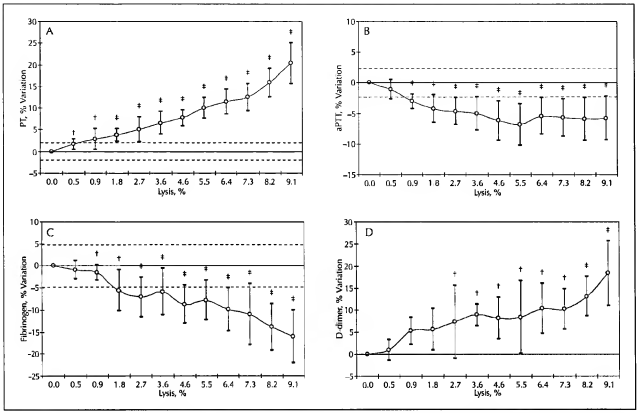
RESULTS

Results of our investigation are synthesized in the Figure. The addition of blood cell lysates generated a consistent and dose-dependent trend toward overestimation of PT (Figure, A) and D-dimer values (Figure, D), whereas aPTT (Figure, B) and fibrinogen (Figure, C) values were substantially decreased when compared with the baseline specimens (no lysate). Statistically significant differences by the Student paired *t* test were observed in samples containing a final lysate concentration of 0.5% for PT, 0.9% for aPTT and fibrinogen, and 2.7% for D-dimer. Percent variations from the baseline value exceeding the current analytical quality specifications for desirable bias were achieved for lysate concentrations of 0.9% (PT and aPTT) and 1.8% (fibrinogen), corresponding to average free plasma hemoglobin concentrations of 1.7 and 3.4 g/L, respectively. There was a roughly linear relationship between the degree of variation and the percentage of lysate in the plasma. However, an unpredictable, sample-specific response was observed for each of the parameters assayed, as shown by the amplitude of the mean coefficients of variation, which ranged from 24% to 28% for PT, from 19% to 20% for aPTT, from 23% to 27% for fibrinogen, and from 54% to 62% for D-dimer. Because the clotting assays were performed at the alternative 570-nm wavelength, these variations were almost referable to the direct effect of hemoglobin, intracellular components, and thromboplastic substances released by damaged cells, rather than to an optical interference.

COMMENT

Hemolysis, causing leakage of hemoglobin and other internal components from the erythrocyte membrane into the surrounding fluid, is usually defined for extracellular hemoglobin concentrations greater than 0.3 g/L (18.8 mmol/L). Hemolysis confers a detectable pink-to-red hue to serum or plasma and becomes clearly visible in specimens containing as low as 0.5% hemolysate.[8] Hemolyzed specimens are a rather frequent occurrence in laboratory practice, and the relative prevalence is described as being as high as 3.3% of all of the samples afferent to a clinical laboratory.[9] Hemolysis, and blood cell lysis in general, are caused by biochemical, immunologic, physical, and chemical mechanisms. In vivo blood cell lysis, which can arise from hereditary, acquired, and iatrogenic conditions (such as autoimmune hemolytic anemia, severe infections, intravascular disseminated coagulation, or transfusion reactions), does not depend on the technique of the healthcare provider; thus, it is virtually unavoidable and not solvable.[10] Conversely, in vitro blood cell lysis might be prevented, because it is usually caused by inappropriate specimen collection, handling, and processing. In the case of specimen collection, hemolysis might result from cumbersome or traumatic specimen collection and processing, such as unsatisfactory phlebotomy attempts, difficulty locating venous accesses, prolonged tourniquet time, wet-alcohol transfer from the skin into the blood specimen, small or fragile veins, missing the vein, syringes or butterfly collection devices, small-gauge needles, partial obstruction of catheters, vigorous tube mixing and shaking, or exposure to excessively hot or cold temperatures, although slow leakage may also occur.[11] Hemolysis and blood cell lysis may not be evident until centrifugation of the whole blood specimen has been performed, exposing the serum or plasma to scrutiny.

Consistent quality specimens can only result from proper training and the knowledge of the factors that can influence laboratory results. Hemolysis, reflecting a more generalized blood cell lysis, is the most frequent reason for specimen rejection, as indicated by the College of



Influence of blood cell lysis on prothrombin time (PT; A), activated partial thromboplastin time (aPTT; B), fibrinogen testing (C), and dimerized plasmin fragment D testing (D-dimer; D). Differences are given in percentage (mean ± SD) from the baseline sample (no lysis). The dashed horizontal lines indicate the current limits of the analytical quality specifications for desirable bias. Statistically significant differences are evaluated by Student paired t *test († P < .05; ‡ P < .01).*

American Pathologists Chemistry Specimen Acceptance Q-Probes study.[12] In fact, the release of hemoglobin and additional intracellular contents from erythrocytes, white blood cells, and platelets into the surrounding fluid might falsely elevate measurable levels of the same substances in serum and plasma, or might cause dilution effects, which may compromise the reliability of laboratory testing.[13] Additionally, plasma hemoglobin might increase the optical absorbance or change the blank value, producing method-dependent and analyte concentration–dependent spectrophotometric interference with common laboratory assays.[9] It is traditionally accepted that both in vivo and, more commonly, in vitro blood cell lysis can cause preanalytical variability. Although the amount of interference will depend on the degree of lysis and on the specificity of the method being used, several laboratory results can be affected, especially potassium, sodium, calcium, magnesium, bilirubin, haptoglobin, total protein, aldolase, amylase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, phosphorus, alkaline phosphatase, acid phosphatase, γ-glutamyl transpeptidase, folate, and iron measurements.[13–15] It is traditionally known that routine coagulation testing might also be influenced by blood cell lysis. The interference is not necessarily only caused by hemoglobin, because many substances are released from blood cells and could influence coagulation assays. In fact, it is more likely that the effects we observed are caused by release of intracellular and thromboplastic substances from either leukocytes or platelets, which are thought to be responsible for shortening the aPTT, as reported by Garton and Larsen[16] However, there is no definitive evidence or agreement in the current literature on this topic, to our knowledge. Among laboratory testing, PT, aPTT, fibrinogen, and D-dimer measurements are thought to be more susceptible to variations in the preanalytical phase.[17] Results of our investigation indicate that a slight lysis, as low as 0.9%, might influence the reliability of some coagulation testing. We are aware that our investigation has 2 major limitations. First, we reproduced in vitro blood cell lysis by freezing blood specimens at −70°C for up to 24 hours. In addition to lysed erythrocytes, hemolyzed specimens encountered in the laboratory practice usually contain destroyed or fragmented platelets and leukocytes. Therefore, our method seems to be a suitable surrogate, although it is not representative of all of the possible events that can induce whole blood lysis in laboratory practice, especially those represented by troublesome blood collection. Second, we evaluated interference on testing of single reagents and with defined analyzers; therefore, our results might not be universally reproducible or transferable to other testing systems. This is particularly true for analyzers that measure the extent of hemolysis by comparing the absorption of samples at 2 wavelengths, or allow performance of coagulation assays at alternative wavelengths, such as 570 nm, for turbid, icteric, and hemolytic samples. The light source of the Beh-

ring Coagulation System photometer is a xenon flasher lamp with broadband emission; an interference filter with an appropriate main wavelength is swung into the beam of the light source to obtain light with the desired wavelength. The instrument does not identify hemolytic specimens with a specific flag. However, in our experience, results obtained by conventional assays were systematically flagged as questionable by the addition of a question mark, starting from a final percentage of lysis ranging from 0.5% to 0.9%.

The issue of hemolysis and blood cell lysis has plagued clinical laboratories and continues to be a growing concern. The most frequent causes, such as sampling errors, are avoided by using standardized materials and methods for the preanalytical processes and by training and individual counseling. Although hemolyzed and unsuitable samples are unlikely to be observed in a problem-free phlebotomy activity, each laboratory should document the procedures that are influenced by blood cell lysis and to what extent they are affected. Some instruments report an ability to correct the results for hemolysis, but after our experience, and, at variance with other observations,[18] we do not recommend hemolysis correction in clinical practice, given the wide interindividual and analytical variations arising from the interference. Therefore, if hemolysis and blood cell lysis results from an in vitro cause, we suggest that the most convenient corrective measure might be free hemoglobin quantification and sample recollection.

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